Human parthenogenetic blastocysts derived from noninseminated cryopreserved human oocytes

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Objective: To report on the development of human parthenogenetic blastocysts and an in vitro attachment that was generated from noninseminated cryopreserved human oocytes for the first time.
Design: Prospective study.
Setting: Department of reproductive medicine in a medical institute in Buenos Aires, Argentina.
Patient(s): Five healthy fertile donors.
Intervention(s): Artificial activation of noninseminated cryopreserved human oocytes after thawing, parthenote culture, and their in vitro attachment.
Main Outcome Measure(s): Survival rate, activation rate, cleavage rate, and blastocyst formation.
Result(s): Thirty-six of 38 cryopreserved noninseminated oocytes survived after thawing (survival rate, 94.7%). Thirty-one of 36 oocytes showed one pronucleus (activation rate, 86.1%). Thirty of 31 cleaved (cleavage rate, 96.8%). Five of 30 showed cavitation (blastocyst rate, 16.7%).
Conclusion(s): Noninseminated cryopreserved human oocytes showed a high survival rate after thawing. They responded very satisfactorily to artificial activation, which was followed by a high rate of parthenogenetic embryos, which can develop into blastocysts. In the future, these could be a new source for development of human parthenogenetic stem cells. (Fertil Steril® 2008;89:943–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Parthenogenesis, oocyte cryopreservation, parthenogenetic activation, parthenogenetic embryos, parthenogenetic blastocysts

Human oocyte cryopreservation now is slowly becoming mainstream in the daily practice of assisted reproductive technology procedures, thanks to its potential therapeutic applications (1–7). It also can be a great source of oocytes for research in different fields.

The therapeutic uses of human embryonic stem cells that are derived from viable pre-embryos are very promising (8–11). However, some countries argue against this usage because of the ethical dilemmas it may cause. One of the alternative protocols proposed is parthenogenesis, in which embryonic development is initiated without sperm contribution. Parthenogenetic activation mainly was studied in experimental species (12–14).

Studies elsewhere have demonstrated the possibility of obtaining stem cells from the parthenogenetic activation of oocytes of nonhuman primates (14). An important point to clarify is that although parthenogenesis is a common method for reproduction among lower organisms, the mammalian parthenote fails to produce a successful pregnancy (15).

Received October 24, 2006; revised and accepted April 4, 2007.
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As far as we know, there have been no reports showing the use of noninseminated cryopreserved human oocytes as a source of oocytes for parthenogenetic development.

The objective of the present study is to report for the first time on the development of human parthenogenetic blastocysts and their in vitro attachment from noninseminated cryopreserved human oocytes.

MATERIALS AND METHODS

Oocytes Donors

The oocytes were provided from five healthy, fertile donors (mean age ± SD was 32.2 ± 3.4).

Informed consent forms approved by the institutional review board of our institution's Human Subjects Research and Ethics Committee were signed by all donors who participated in the present study.

The donors were given a complete medical checkup, consisting of a detailed personal and family medical history, psychological evaluation, gynecological exam, and ultrasound scan.

A complete blood test was performed to check for human immunodeficiency virus, hepatitis, and syphilis, and a karyotype analysis was performed, as well as hormonal testing, to ensure the viability of the potential donors.

After oocyte recovery, the donors were periodically and carefully checked, and they were discharged after obtaining a normal ultrasound post-menstruation.

Ovarian Stimulation

Controlled ovarian hyperstimulation was initiated in all patients by using the GnRH analogue leuprolide acetate (given SC) from the midluteal phase of the previous cycle. Gonadotropin therapy was performed by using recombinant FSH (Puregon, Organon Argentina, Buenos Aires, Argentina).

Regular monitoring was performed by using serial transvaginal ultrasonography and serum E_2 levels, as published elsewhere (6).

Ovulation was induced by using hCG (10,000 IU, administered IM; Pregnyl, Organon Argentina), when at least two follicles of $\geq$18 mm in diameter were observed, with adequate E_2 concentrations. The mean date of hCG administration was on day 12 of the cycle. Transvaginal ultrasound–guided oocyte retrieval was performed 34 hours later.

Oocyte Selection

Once the oocytes were identified in the follicular fluids, they were transferred to human tubal fluid medium that was supplemented with 0.5% human serum albumin (Irvine Scientific, Santa Ana, CA) and were cultured in a 5% CO_2 atmosphere at 37°C for 3 hours.

Complete removal of cumulus and corona cells was performed by using a brief exposure to hyaluronidase (80 IU/mL; Sigma Chemical, St. Louis, MO) and mechanical manipulation with fine-bore glass pipettes. Only oocytes showing an extruded polar body (that therefore presumably were at the metaphase II stage) were frozen after culture for about 4 hours from retrieval.

Freezing and Thawing Procedure

Thirty-eight noninseminated fresh human metaphase II oocytes were cryopreserved by using the 1,2-propanediol slow-freeze–rapid-thaw method with 0.3 M sucrose (16).

All gametes were washed in Dulbecco's phosphate-buffered saline solution (1×; Irvine Scientific), supplemented with 30% serum substitute supplement (Irvine Scientific). After that, they were placed in 1.5 M 1,2-propanediol that was supplemented with 30% serum substitute supplement for 10 minutes. Afterward, the oocytes were transferred to the loading solution containing 1.5 M 1,2-propanediol, 30% serum substitute supplement, and 0.3 M sucrose; loaded into plastic straws; and placed into a biological freezer.

The temperature was gradually lowered from 16°C to –6.5°C at a rate of –2°C/min, at which point manual seeding was performed. The temperature continued to be lowered at a rate of 0.3°C/min, until –35°C was reached. The straws finally were plunged into liquid nitrogen (−196°C) and stored for later use.

The thawing procedure was performed at room temperature. The straws were air-warmed for 30 seconds and then plunged into a 30°C water bath for 40 seconds. The cryoprotectant was removed by transferring the oocytes through decreasing concentrations of propanediol solution (1 M to 0.5 M in each) containing 0.3 M sucrose, followed by a dilution of 0.3 M sucrose alone; finally, they were washed in Dulbecco's phosphate-buffered saline solution (1×). All the solutions were supplemented with 30% serum substitute supplement. Finally, the viable oocytes were cultured in human tubal fluid medium that was supplemented with 0.5% human serum albumin at 37°C in an atmosphere of 5% CO_2 in air for $\geq$3 hours before activation.

Artificial Oocyte Activation

After thawing, 36 noninseminated oocytes were parthenogenetically activated by using a combination of ionomycin (CalBiochem, San Diego, CA) and 6-dimethylamino purine (Sigma, St Louis, MO). They were exposed to manipulation medium (human tubal fluid–HEPES) with 10 uM of ionomycin for 6 minutes at 37°C in room air. The oocytes then were moved to a K+-modified simplex optimized medium (KSOM) culture medium (Specialty Media) + human serum albumin with 2 mM of 6-dimethylamino purine for 3 hours at 37°C, 6% CO_2 in air. Parthenogenetic embryos were changed to KSOM + human serum albumin medium for 72 hours and then to another fresh KSOM + human serum albumin media drop at 37°C, 6% CO_2 in air. Parthenogenetic blastocysts were plated on top of mitotically inactivated human umbilical cord fibroblasts, with the whole embryo culture method or partial embryo culture described by Kim et al. (17), depending on the morphology of the blastocyst and the appearance of the inner mass cells (IMCs). The medium used was Knockout-Dulbecco's Modified Eagle Medium (KO-DMEM; GIBCO Invitrogen, Grand Island, NY) supplemented with 15% fetal calf serum (GIBCO), penicillin/streptomycin, 1× nonessential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 4 ng/mL basic Fibroblast Growth Factor.

All parthenotes were checked periodically to monitor their growth and attachment, and the medium was renewed during the checks. Photographic registers were made.

RESULTS

Thirty-six of 38 cryopreserved noninseminated oocytes survived after thawing (survival rate, 94.7%).

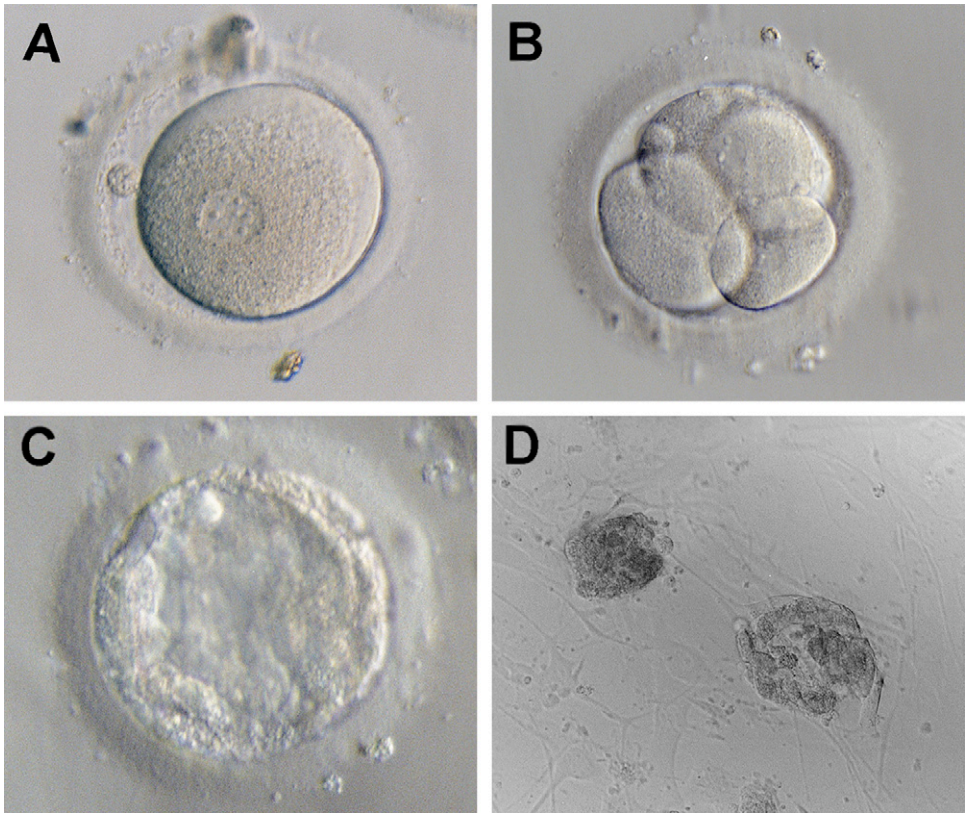
Thirty-one of 36 oocytes showed only one pronucleus (activation rate, 86.1%; Fig. 1A). Thirty of 31 cleaved (cleavage rate, 96.8%; Fig. 1B).

After activation, three embryos on day 6 and two embryos on day 7 showed cavitation. According to the classification of Gardner et al. (18), the quality of the blastocysts mainly was poor (Fig. 1C). The blastocyst rate was 16.7%.

Human parthenogenetic blastocysts were plated. After plating, one blastocyst exhibited incipient attachment, and the other showed complete attachment (Fig. 1D).

FIGURE 1

Oocyte photomicrographs. (**A**) Pronucleus. (**B**) Cleavage. (**C**) Poor-quality blastocysts. (**D**) Blastocyst attachment.



Polak de Fried. Parthenogenetic blastocysts from frozen oocytes. Fertil Steril 2008.

No further development was observed.

Fifteen nonevolutive cleaved parthenotes were plated on day 9 after activation. Six of 15 showed attachment.

After 63 days in co-culture, three of three parthenotes showed attachment. No signs of growth, measured either in cell number and/or colony size, were observed.

DISCUSSION

Many published studies have reported the use of cryopreserved oocytes that had been frozen for different purposes (1, 2, 6, 7, 19–24).

During recent years, the main challenge in this area has been to improve the survival rate of cryopreserved oocytes.

The oocyte survival rate after cryopreservation has been reported to be variable (27%–64%). In a study published elsewhere, we reported a survival rate of 30% when using 0.1 M sucrose (2). In another recent study, other investigators reported a survival rate of 37%, also using 0.1 M sucrose (24).

Because Fabbri and co-investigators reported that a higher concentration of sucrose (0.3 M) dramatically improved the survival rate of cryopreserved oocytes (83%) (16), other investigators repeated the same procedure, with similar results (23).

In the present study, we used 0.3 M sucrose to cryopreserve 38 fresh, noninseminated oocytes, and the survival rate after thawing was 94.7%. These results are further evidence of the beneficial effects of higher concentrations of sucrose on the survival rate after thawing of cryopreserved oocytes.

To date, parthenogenetic activation of oocytes was performed in most mammals, including mice, goats, cows, monkeys, and human beings (12–15, 25–33).

Instead of using fertilized and viable embryos for research, this procedure could give scientists the opportunity to work in this field in countries that have restrictions on the use of human gametes and human embryos.

Most of the published literature regarding parthenogenetic activation in human beings is based on the use of oocytes that failed to fertilize after IVF-ICSI procedures. Hence, they were unfertilized human aged oocytes that were exposed to different activation techniques (25, 26). We consider that

this is not the most appropriate material to evaluate parthenogenetic activation in human oocytes, because they were in contact with sperm and also experienced adverse aging effects. In our laboratory, 164 of 197 fresh human oocytes were activated (activation rate, 83.25%) to compare different activation techniques (Polak de Fried, unpublished data).

In the present study, the source of the human oocytes for parthenogenetic activation was noninseminated, cryopreserved human oocytes, and the parthenogenetic activation rate was 86.1%, the cleavage rate was 96.8%, and the cavitation rate was 16.7%. According to this study, the cryopreservation procedure does not affect the parthenogenetic activation rate and the late development of the parthenotes.

Freezing oocytes could be a good technique for the preservation of female fertility that has been reduced as the result of medical treatments or as a result of the detrimental effect of aging. In the future, cryopreserved human oocytes also could be a valuable source of stem cells for therapeutic applications that may produce stem cells with immunological competence. In addition, their parthenogenetic activation could provide the opportunity to work in this field for scientists in countries that have restrictions on the use of human gametes and embryos. A great number of in-depth and ongoing studies are needed to provide more evidence in this respect.

As far as we know, this is the first report about the parthenogenetic activation of noninseminated cryopreserved human oocytes.

The high survival rate allowed for significant parthenogenetic formation and blastocyst development and attachment. Currently, we continue the experiments because our goal is to achieve the possibility of obtaining stem cells from this source.

Acknowledgments: The authors thank everyone who collaborated for the present study. Special thanks go to Gabriela Arenas, M.Sc. (for her technical help), to Florencia Luna, Ph.D. (for correcting the donors' informed consent forms as well as supervising all topics related to ethical concerns), and to Virginia Cafiero, M.A. (for the psychological guidance given to the donors).

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